Application of Anti-Methotrexate Fab Fragments for the Optimization of Intraperitoneal Methotrexate Therapy in a Murine Model of Peritoneal Cancer

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Received 7 January 2005; revised 11 May 2005; accepted 12 May 2005

Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jps.20422

ABSTRACT: Anti-drug antibodies may be used to impart regio-specific alterations in drug disposition, potentially enhancing the therapeutic selectivity of intracavitary chemotherapy. In the present study, we tested the hypotheses that systemic therapy with anti-methotrexate antibodies would allow increases in the maximum tolerated dose of intraperitoneal methotrexate (MTX) and allow increases in the therapeutic efficacy of intraperitoneal MTX in a murine model of peritoneal cancer. Monoclonal anti-MTX Fab antibody fragments (AMF) were produced, purified, and characterized. AMF pharmacokinetics were determined following i.v. bolus injection (0.4 g/kg) and s.c. bolus injection (0.4, 0.8, 2.2 g/kg). MTX efficacy was investigated in mice bearing peritoneal sarcoma 180 tumors, following administration of MTX via 72 h i.p. infusion at 1.9, 2.8, 3.8 mg/kg, and following combination therapy of 7.5 or 10 mg/kg i.p. MTX (72 h infusion) and 4.2 g/kg s.c. AMF. The mean terminal half-life of AMF was found to be 10.9 ± 3.3 h and was not dose-dependent, and s.c. bioavailability was 28% ± 7% at 2.2 g/kg. In mice bearing peritoneal tumors, the maximally tolerated dose of i.p. MTX increased from 1.9 mg/kg (following i.p. MTX alone) to 10 mg/kg (with co-administration of s.c. AMF). Median survival times for saline-treated control animals and animals receiving i.p. MTX (1.9, 2.8, 3.8 mg/kg) were 9, 12, 10, and 7 days, respectively. However, for animals receiving combination therapy with i.p. MTX 7.5 or 10 mg/kg and 4.2 g/kg s.c. AMF, median survival time increased to 17 and 14 days, respectively. As such, the present data suggest that systemic administration of AMF may allow increases in the maximally tolerated dose of i.p. MTX, and allow increases in the therapeutic efficacy of i.p. MTX chemotherapy of peritoneal tumors.
 J Pharm Sci 94:1957–1964, 2005
Keywords: antibody; drug targeting; cancer; chemotherapy

INTRODUCTION

This research group has been investigating a targeting approach that attempts to improve the selectivity of intraperitoneal chemotherapy for peritoneal tumors.[1,2] The approach combines i.p. chemotherapy with systemic administration of anti-drug antibodies to impart site-selective alterations in drug disposition. It is hypothesized that the presence of anti-drug antibodies in the systemic circulation would lead to rapid complexation of the drug diffusing out of the peritoneum and entering the blood, potentially reducing systemic free drug exposure and minimizing extravascular drug distribution.

Previous studies in rats demonstrated that i.v. administration of anti-methotrexate antibody fragments with simultaneous i.p. administration of methotrexate (MTX) led to reductions in peak plasma concentrations of unbound MTX and in the

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Journal of Pharmaceutical Sciences, Vol. 94, 1957–1964 (2005)

EXHIBIT 2

area under the unbound MTX plasma concentration versus time curve, without significantly altering peritoneal MTX exposure.[2] Additionally, AMF enhanced the urinary elimination of MTX and decreased the apparent volume of MTX distribution. In more recent toxicity studies conducted in mice, i.v. AMF therapy was found to decrease MTX-induced body weight loss and MTX-related mortality, following i.p. MTX administration.[3]

In this report, we have tested the hypothesis that AMF will allow increases in the maximally tolerated dose of i.p. MTX, and we investigated the anti-tumor efficacy of MTX and MTX/AMF combination therapy in a peritoneal tumor model in mice. Additionally, the pharmacokinetics of AMF were investigated in mice following i.v. and s.c. administration.

METHODS

Materials

MTX (>99.9% purity), xylazine, Tween-20, bovine serum albumin, and Fab specific goat anti-mouse-antibody-alkaline phosphatase were obtained from Sigma (St. Louis, MO). ALZET® microosmotic pumps (1003D), for 3 day constant-rate infusion, were obtained from Alza Corporation (Palo Alto, CA). Swiss Webster male mice, 5–6 week-old (20–25 g) were obtained from Harlan Sprague–Dawley, Inc. (Indianapolis, IN), and housed on a standard light/dark cycle, with continuous access to food and water. Cell culture media (RPMI 1640), certified fetal bovine serum, and gentamicin were obtained from Invitrogen Corporation (Grand Island, NY). Sarcoma 180 cells were obtained from American Type Cell Culture (Manassas, VA).

AMF Production

AMF was produced, purified, and characterized as reported previously.[3] Briefly, anti-MTX IgG was produced from hybridoma cells (developed in this laboratory), purified by affinity chromatography, and digested with papain. AMF was purified from undigested IgG, Fc fragments, and secondary digestion products through hydroxyapatite chromatography (BioRad Laboratories, Hercules, CA). Concentrated AMF in PBS (pH 7.4) was passed through Detoxi endotoxin removing gel (Pierce, Rockford, IL) at least three times prior to use. Endotoxin concentrations in antibody solutions used *in vivo* were lower than 20 ng/mL; as such, animals were exposed to doses of endotoxin that were well below those associated with toxicity in mice.[4]

ELISA Assay for AMF

Assays were conducted using Maxisorp 96-well microplates (Nunc, Rosklide, Denmark) as previously described.[5] Briefly, bovine serum albumin-methotrexate conjugates (10 μg/mL in 0.02M Na_2HPO_4, no pH adjustment), 0.25 mL, was placed into each assay well, and assay plates were incubated overnight at 4°C. Following incubation, plates were washed three times with a phosphate buffer (PB-Tween) consisting of 0.05% Tween 20 and 0.02M Na_2HPO_4 (no pH adjustment), and rinsed twice with distilled water. Diluted plasma samples and standards were added and plates were incubated 2 h at room temperature. Standards were made by diluting a stock concentration of purified AMF to concentrations of 0, 25, 50, 100, 250, and 350 ng/mL with phosphate buffered saline (pH 7.4) containing 1% blank mouse plasma (Hilltop Lab Animals, Inc., Scottdale, PA). After incubation, the plate was washed with PB-Tween, rinsed with distilled water, and a secondary antibody conjugate was applied (0.25 mL, Fab specific goat anti-mouse-antibody-alkaline phosphatase, diluted 1:500 in PB Tween and 1% bovine serum albumin). After incubating with the secondary antibody at room temperature for 1 h, plates were washed with PB-Tween, rinsed with distilled water, and *p*-nitro phenyl phosphate (Pierce) was applied (4 mg/mL, 0.2 mL, pH 9.8). Plates were analyzed with a microplate reader (Spectra Max 340PC, Molecular Devices, Sunnyvale, CA), which assessed the change in absorbance at 405 nm with time (dA/dt). Standard curves, using standards run on each assay plate (0–350 ng/mL), were created to relate AMF concentration to dA/dt. The limit of quantitation of this assay was 25 ng/mL; intra- and inter-assay variability were found to be less than 13%.

AMF Pharmacokinetics

AMF was administered via i.v. bolus injection (0.4 g/kg) or via s.c. bolus injection (0.4, 0.8, and 2.2 g/kg) to groups of three Swiss Webster mice. Animals receiving i.v. AMF were instrumented with jugular vein cannulas 3 days prior to experimentation. Following i.v. bolus injection,

blood samples (10–20 μL) were collected from the saphenous vein at 0.25, 0.5, 1, 2, 3, 6, 9, 24, and 48 h. After s.c. bolus injection, blood samples were collected at 0.5, 1, 2, 3, 6, 12, 24, and 48 h. Plasma was obtained after centrifuging the blood at 10000*g* for 2 min, and AMF concentration was determined using the ELISA. The area under the plasma concentration versus time curves, AUC, was determined by non-compartmental analysis with WinNonlin software (Pharsight Corporation, Mountain View, CA), and bioavailability (F) was calculated as $F = (AUC_{sc}/AUC_{iv}) \times (Dose_{iv}/Dose_{sc})$.

Toxicity Studies

In previous investigations of MTX-induced toxicity in Swiss–Webster mice, the maximally tolerated i.p. dose of MTX, infused over 72 h, was 3.8 mg/kg.[6] In this same investigation, 72 h, infusion of 5 and 10 mg/kg MTX led to a mean nadir weight-loss of 15.6% ± 5.3% and 23.8% ± 3.3%, respectively. In the present study, MTX-induced toxicity was investigated in two groups of 4–5 animals treated with 10 mg/kg i.p. MTX, infused over 72 h, with or without coadministration of s.c. AMF (4.2 g/kg, administered as two injections of 2.1 g/kg, separated by 31 h). MTX infusions were delivered via osmotic pumps attached to peritoneal cannulas. Briefly, animals were instrumented with osmotic pumps (pre-equibrated overnight at 37°C in 0.9% sodium chloride solution) under ketamine/xylazine (100/10 mg/kg) anesthesia. Pumps were placed on the dorsal side near the shoulder blade, cannulas were tunneled subcutaneously, and inserted into the peritoneal cavity through a small incision made through the abdominal muscle layer. Animal body weight was recorded daily. Weight loss at nadir is reported as a percentage of animal weight just prior to pump implantation. In cases of animal death, weight loss is reported as the maximal percent weight loss prior to expiration.

Efficacy Studies

MTX efficacy was investigated in Swiss Webster mice inoculated i.p. with sarcoma 180 cells. Prior to injection, sarcoma 180 cells were maintained in culture with RPMI 1640 media containing 20 nM folic acid, 10% fetal bovine serum and 100 μg/mL gentamicin. One day prior to the initiation of drug treatment, cells were washed, suspended in sterile 0.9% sodium chloride, and injected into animals i.p. (10^6 cells in 0.5 mL). MTX was administered via constant-rate i.p. infusion, as described above, at doses of 1.9, 2.8, and 3.8 mg/kg (n = 10 animals/group). Additionally, MTX/AMF combination therapy was investigated where MTX was administered i.p. over 72 h (7.5 or 10 mg/kg) with s.c. administration of AMF (4.2 g/kg, administered as two 2.1 g/kg s.c. doses separated by 31 h, $n = 5$). Animal weight was recorded daily, and animals were sacrificed when body weight increased to 120% of animal weight on the day of tumor inoculation. Following sacrifice, animals were inspected for evidence of peritoneal tumors. The maximally tolerated dose of MTX in tumor bearing mice was defined as the highest dose for which no deaths occurred prior to the first death of control animals (i.e., animals inoculated with tumor, but not treated with drug). The percent increase in life span (% ILS) was calculated as: % ILS = (median survival time in treated animals – median survival time in control animals)/median survival time in control animals ×100.

Statistics

Data are expressed as mean and the standard deviation. One-way analysis of variance with Bonferroni post-test was applied to compare half-lives. Unpaired two-tailed Student's t-tests were applied to compare the mean nadir body weight loss. Mortality rates were compared using the Binomial proportion test. Survival curves were compared for significance with the logrank test and hazard ratio. Statistical analysis was performed using InStat and Prism software (Graph Pad Software, Inc., San Diego, CA).

RESULTS

AMF Pharmacokinetics

The AMF concentration time profile following intravenous and subcutaneous administration is shown in Figure 1. Non-compartmental analysis of the data is summarized in Table 1. The terminal half of AMF was found to be 10.9 ± 3.3 h, and was not dependent on dose (in the dose range of 0.4 to 2.2 g/kg, $p > 0.05$), or on the route of administration (i.v. or s.c., $p > 0.05$). AMF s.c. bioavailability was low and variable, ranging from 0.09 ± 0.01 at 0.8 g/kg to 0.28 ± 0.07 at 2.2 g/kg.

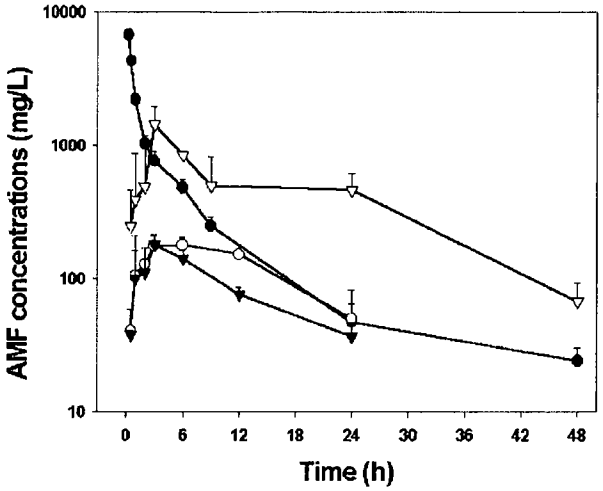


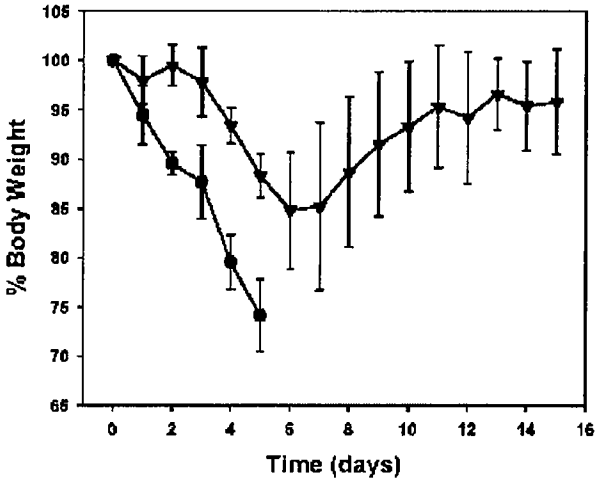
Figure 1. AMF pharmacokinetics in mice. Mean plasma concentration of AMF following i.v. 0.4 g/kg bolus injection (●), and following s.c. bolus injections of 0.4 g/kg (○), 0.8 g/kg (▼), and 2.2 g/kg (▽). The mean and standard deviations for AMF concentration ($n = 3$) are represented as symbols and error bars.

Toxicity Studies

Animal body weight versus time is shown in Figure 2A. For animals receiving 10 mg/kg i.p. MTX with 4.2 g/kg AMF, mean weight loss at nadir was found to be (16.6% ± 5.8%, $n = 4$), which was significantly different that observed for animals receiving 10 mg/kg i.p. MTX alone (25.8% ± 3.7%, $n = 5$, $p < 0.05$). Treatment-associated mortality was markedly different between the two treatment groups; all animals receiving MTX alone died, whereas all animals receiving MTX/AMF combination therapy survived ($p < 0.01$).

MTX Efficacy in Peritoneal Tumor Bearing Mice

The survival curves for tumor bearing control animals and for tumor bearing animals treated



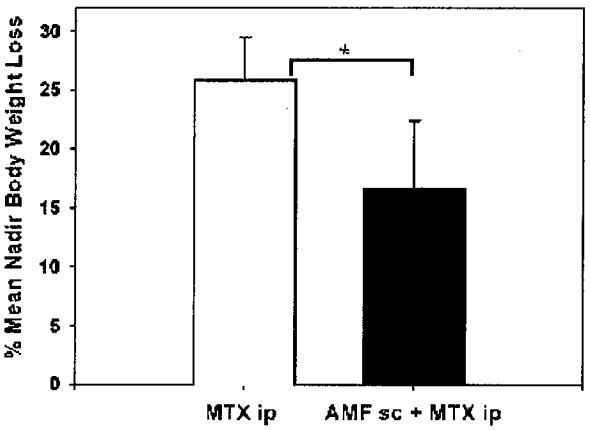


Figure 2. Dose escalation of i.p. MTX therapy with s.c. AMF. Animals were treated with 10 mg/kg of i.p. MTX over 72 h infusion with or without coadministration of 4.2 g/kg of AMF given as two s.c. bolus injections of 2.1 g/kg (separated by 31 h). (A) MTX-induced changes in body weight without AMF (●) and with AMF (▼). All animals treated with MTX alone ($n = 5$) died whereas all animals treated with MTX/AMF ($n = 4$) survived. (B) MTX-induced mean nadir body weight loss with and without AMF ($p < 0.05$).

Table 1. Non-Compartmental Analysis of AMF Pharmacokinetics in Mice*

	i.v. 0.4 g/kg	s.c. 0.4 g/kg	s.c. 0.8 g/kg	s.c. 2.2 g/kg
AUC (g/L × min)	840 ± 2.6	225 ± 63	157 ± 14.3	1300 ± 310
F	—	0.27 ± 0.07	0.09 ± 0.01	0.28 ± 0.07
$t_{1/2}$	8.8 ± 1.4	8.5 ± 3.7	9.5 ± 2.3	11.9 ± 2.6
CL (mL/kg/h)	28.6 ± 0.1	—	—	—
Vss (mL/kg)	191.6 ± 41.4	—	—	—

*AUC, area under the plasma concentration time curve from 0 to infinity; CL, systemic clearance; Vss, volume of distribution at steady-state; and λ, negative terminal slope of ln (AMF concentration) versus time curve. Parameter values were obtained using WinNonlin. $t_{1/2}$, terminal half-life was estimated as 0.693/λ and F, the fraction absorbed on s.c. administration was calculated as (s.c. AUC/ i.v. AUC) × (i.v. dose/s.c. dose).

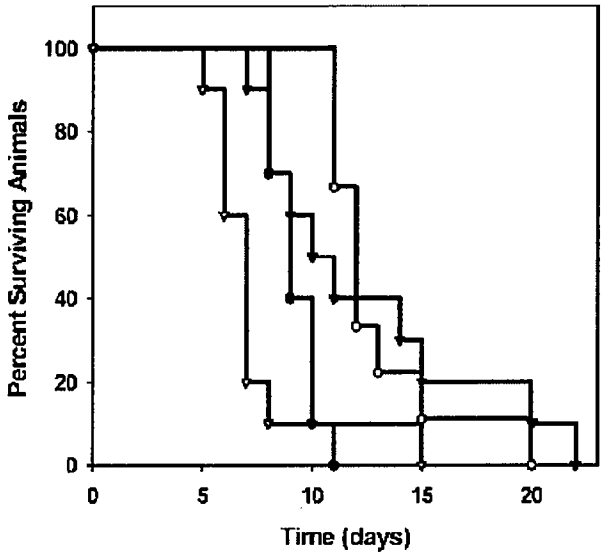


Figure 3. MTX efficacy in mice bearing peritoneal tumors. Each animal was inoculated with 10^6 sarcoma 180 cells (in 0.5 mL of sterile 0.9% NaCl) in the peritoneum (day 0). Treatment was initiated the next day. Control animals were treated with i.p. bolus injection of sterile 0.9% NaCl (0.5 mL). MTX treated animals received MTX as i.p. 72 h constant-rate infusion at 1.9, 2.8, and 3.8 mg/kg. The survival time was defined as the time to death or the time to reach 120% body weight. Survival curves for (●) control animals and MTX treated animals at (○) 1.9 mg/kg, (▼) 2.8 mg/kg, and (▽) 3.8 mg/kg. Survival in animals treated with 1.9 mg/kg MTX was significantly different from the control animals ($p < 0.001$). Survival of animals treated with 3.8 mg/kg MTX was significantly reduced relative to the controls ($p < 0.05$).

with i.p. MTX (1.9, 2.8, and 3.8 mg/kg) are shown in Figure 3. The median survival times and % ILS for all the treatment groups are summarized in Table 2. The highest tolerated MTX dose was observed to be 1.9 mg/kg, and animal survival following this dose of MTX was significantly increased from that observed for control animals ($p < 0.001$). Following 2.8 mg/kg animal survival was not significantly different compared to that of control animals ($p > 0.05$), and following 3.8 mg/kg animal survival was significantly reduced relative to the controls ($p < 0.05$).

Following combination therapy with i.p. MTX (7.5 or 10 mg/kg) and s.c. AMF (4.2 g/kg), median survival time was increased relative to the control group and the MTX treated groups (Fig. 4). Relative to the controls, animal survival was significantly improved following combination therapy with 7.5 mg/kg i.p. MTX and s.c. AMF ($p < 0.001$) and following the combination of 10 mg/kg i.p. MTX and s.c. AMF ($p = 0.046$).

DISCUSSION

Intraperitoneal chemotherapy has been investigated as a means of improving the selectivity of chemotherapy of intra-abdominal tumors (e.g., ovarian, gastro-intestinal, pancreatic, and colorectal cancers).[7–9] Much of the interest in i.p. chemotherapy stems from predictions made by Dedrick et al.,[10] which suggested that i.p. drug administration would allow much greater peritoneal drug exposure relative to systemic drug exposure. Although clinical pharmacokinetic studies supported the predictions of Dedrick et al., the extent of peritoneal drug exposure afforded by i.p. chemotherapy appears to be insufficient to alter the site-specificity of chemotherapeutic cytotoxicity, as systemic toxicity remains dose-limiting, and only modest improvement in therapeutic benefit has been observed following i.p. chemotherapy.[11]

Dedrick et al.[10] also proposed that the selectivity of i.p. chemotherapy may be enhanced through

Table 2. Summary of MTX Efficacy in Mice Bearing Peritoneal Tumors

Dosing Protocol[a]	N	MST (Days)	% ILS	% Toxic Deaths
Control	10	9	—	—
MTX 1.9 mg/kg	9	12	33	0
MTX 2.8 mg/kg	10	10	11	10
MTX 3.8 mg/kg	10	7	—	40
Sc AMF + MTX 10 mg/kg	5	14	55	0
Sc AMF + MTX 7.5 mg/kg	5	17	89	0

[a]Dosing protocol, control animals received bolus injection of sterile 0.9% NaCl; MTX was infused at constant-rate over 72 h; AMF was given as two s.c. bolus injections of 2.1 g/kg at 31 h interval; N, number of animals; MST, median survival time; % ILS, percent increase in life span calculated as (Treated MST − Control MST)/Control MST × 100. Toxic deaths, percent of deaths prior to the lowest survival time observed for control animals (i.e., 7 days).

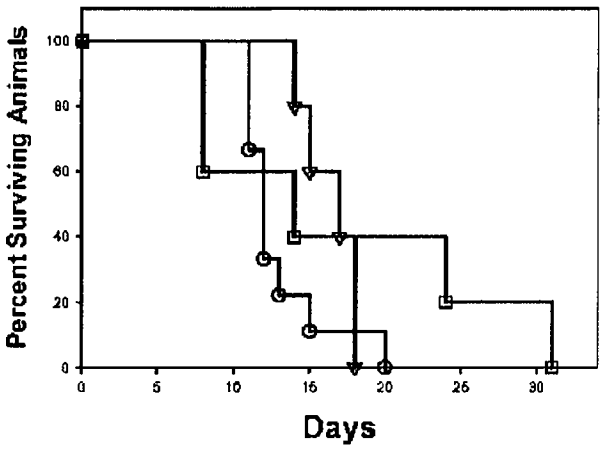


Figure 4. Comparison of MTX efficacy with or without co-administration of anti-MTX Fab fragments. Each animal was injected with 10^6 sarcoma 180 cells (in 0.5 mL sterile 0.9% NaCl) into the peritoneum (day 0). Treatment was initiated the next day. MTX efficacy at the maximally tolerated dose of i.p. MTX (1.9 mg/kg) was compared to that observed following AMF/MTX combination therapy of i.p. MTX, 72 h infusion (7.5 or 10 mg/kg), and s.c. AMF (given as two s.c. bolus injections of 2.1 g/kg). The percent surviving animals treated with (○) 1.9 mg/kg MTX alone, (▽) 7.5 mg/kg MTX + 4.2 g/kg AMF and (□), and 10 mg/kg MTX + 4.2 g/kg AMF.

systemic administration of antidotal agents capable of antagonizing drug that diffuses out of the peritoneal cavity. The combination of i.p. chemotherapy and i.v. administration of antidotes has been investigated with a few drugs, for example: cisplatin (with systemic administration of sodium thiosulfate), mafosfamide (with systemic administration of cysteiene), and neocarzinostatin (with systemic administration of tiopronin).[12–14] However, such combinations have found relatively little application in the clinical treatment of intra-abdominal cancers, perhaps due to slow rates of drug inactivation *in vivo*,[15,16] or perhaps due to observations of decreased drug efficacy (presumably owing to neutralization of drug in the peritoneum).[14]

This research group has been interested in utilizing anti-drug antibodies and antibody fragments as neutralizing agents to reduce systemic toxicities associated with i.p. chemotherapy.[1,2] Antibodies may be raised to bind many drugs with high affinity and with high rates of association; additionally, due to their polar nature and large molecular size, antibodies would be expected to exhibit low rates of entry into the peritoneal cavity. Several preclinical reports have shown that anti-chemotherapeutic antibodies may be used to reduce drug-related mortality and drug-induced body weight loss;[17–19] however, the application of anti-drug antibodies to increase the selectivity or efficacy of i.p. chemotherapy of peritoneal tumors has not yet been investigated.

To develop this targeting strategy, initial preclinical pharmacokinetic studies were conducted with i.p. digoxin/i.v. anti-digoxin Fab and i.p. MTX/i.v. anti-MTX polyclonal antibodies.[1,2,20] These studies demonstrated that systemic antibody administration could reduce systemic tissue exposure to drug, and reduce systemic toxicity, without altering peritoneal drug exposure. Murine anti-MTX monoclonal antibodies and Fab fragments were recently developed in this laboratory,[3] which has now allowed rapid generation of large quantities of antibody, and thereby enabled investigation of the hypotheses that systemic administration of anti-drug antibodies would increase the maximal tolerated i.p. chemotherapeutic dose, and increase the efficacy of i.p. chemotherapy of peritoneal tumors.

Testing these hypotheses required the administration of high doses of anti-MTX Fab. Due to technical difficulties associated with intravenous administration of large doses of antibody (e.g., limited solubility of antibody, limited capacity of infusion pumps, etc.), pharmacokinetic studies were conducted to assess AMF bioavailability following s.c. administration. AMF bioavailability was found to be similar after s.c. administration of 0.4 and 2.2 g/kg ($27\% \pm 7\%$ and $28\% \pm 8\%$), suggesting dose-independent bioavailability. Although s.c. AMF bioavailability was far from complete, it was deemed to be sufficiently high to allow testing AMF/MTX combination therapy with s.c. AMF administration. Notably, AMF bioavailability was found to be significantly lower following s.c. administration of 0.8 g/kg relative to that observed following doses of 0.4 and 2.2 g/kg. This apparent difference in bioavailability may be an artifact, perhaps related to the high variability observed in AMF disposition, and the small sample sizes used in this pilot study ($n = 3$). Additional pharmacokinetic studies will be conducted to develop a comprehensive characterization of AMF disposition, and to assist in future optimization of AMF/MTX combination therapy.

Prior to *in vivo* investigation, dosing regimens for AMF/MTX combination therapy were evaluated via computer simulations conducted with a pharmacokinetic-pharmacodynamic model.[3] These

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